Nosema tephrititae sp. n., A Microsporidian Pathogen of the Oriental Fruit Fly, *Dacus dorsalis* Hendel[1]

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The Oriental fruit fly, *Dacus dorsalis* Hendel, one of Hawaii's most euryphagous agricultural pests, was first recorded in Hawaii from specimens reared from mango (*Mangifera indica* L.) in May 1946 (Fullaway, 1947). Within a few years *D. dorsalis* populations reached very destructive levels. By August 1947, the Oriental fruit fly was found on all major islands comprising the (then) Territory of Hawaii (Anonymous, 1948). A concerted effort involving many agencies for the study and control of this pest was initiated.

In 1951 the USDA Fruit Fly Laboratory in Honolulu, which was mass-rearing *D. dorsalis*, found an apparently new microsporidian pathogen infecting the larvae in the laboratory (Finney, 1951). This microsporidian was tentatively assigned to the genus *Nosema* Naegeli by Dr. E. A. Steinhaus. Since this microsporidian from *D. dorsalis* was apparently a new species, this study was conducted to obtain information in its biology and its effects on the host.

MATERIAL AND METHODS

Nosema spores were originally obtained in 1961 from diseased *Dacus cucurbitae* Coquillett reared at the Fruit Fly Laboratory. To increase the stock inoculum, *D. dorsalis* larvae were infected with the *Nosema* by adding the spores directly to the medium. These larvae were allowed to pupate in sand. Four days later, the pupae were sifted out and macerated in a sterile mortar. A thick homogenate was made by adding distilled water. This homogenate was filtered through organdy, further diluted, concentrated and washed by differential centrifugation. The spores were resuspended in sterile distilled water and stored at 6 °C. Spore concentration was determined by counting the spores in a Petroff-Hausser bacteria counter.

To utilize hosts that were uniform in age, only larvae derived from eggs deposited within a 2 to 3 hour period were used in these tests. One hundred eggs were placed on a moist strip of filter paper and then placed on the rearing medium. The standard fruit fly medium (Mitchell, *et al.*,

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1965) was utilized to which 0.3% by weight of both sodium benzoate and methylparaben powder, USP (Robinson Lab., San Francisco, Calif.) were added as mold inhibitors. The larvae were reared in pyrex petri bottoms (90 mm × 10 mm) containing 80 g of larval medium. Organdy, secured by a rubber band, was used to cover the rearing container.

The larvae were treated by incorporating 1 ml of the stock inoculum (8.9×10^8 spores/ml) into the larval medium. Since the immatures fed on the treated medium from the moment of eclosion until they were removed, they were exposed to the pathogen during the entire larval period. The controls were handled in a identical manner except that 1 ml of sterile distilled water was added to medium instead of the spore suspension.

Studies were conducted at ambient room temperature and humidity. The average temperature during the study period was 28.3°C. with a mean maximum of 29.4°C. and a mean minimum of 27.3°C. The relative humidity averaged 67% with a mean maximum of 77% and a mean minimum of 58%.

Since the infection was usually detected approximately 72 hours after egg hatch, larvae were sacrificed at 6-hour intervals from both treated and control groups beginning at that hour. These larvae were examined for gross external symptoms, and the larger 3rd instar larvae were dissected to study the gross pathology of the internal organs. Wet mounts were also prepared and observed under a phase microscope for the presence of the pathogen. Some of the slides were stained with Giemsa and checked.

For the histopathological studies, larvae were fixed in Carnoy's (6: 3: 1) for 3 hours and prepared for sectioning using the technique described by Smith (1943). Saggital sections were cut at 8 to 10 μ and were stained using the Feulgen reaction with Schiff's de Tomasi reagent as described by Pearse (1960). Sections were counterstained for 5 sec. in 0.1% light green SF yellowish in 95% ethanol.

Infectivity studies were conducted with several dipterous and a lepidopterous species.

Results and Discussion

Identification of the Pathogen. Although several species of *Nosema* have been recorded from dipterous hosts, all of these hosts were in the Suborder Nematocera (Weiser, 1961; Thomson, 1960; Kellen, *et al.*, 1967) with the exception of *N. kingi* Kramer and its cyclorrhaphan host, *Drosophila willistoni* Sturtevant (Kramer, 1964; Burnett and King, 1962). The species of *Nosema* infecting *D. dorsalis*, the 2nd recorded from a cyclorrhaphan, was compared with *Nosema* species having dipterous hosts. Since it was found to differ from other *Nosema* species in several attributes (Table 1), it was evident that this *Nosema* infecting *D. dorsalis* was a new species.

Diagnosis. **Nosema tephrititae** sp. n.

Host. *Dacus dorsalis* Hendel, *D. cucurbitae* Coquillett and *Ceratitis capitata* (Wiedemann)

Locality. Univ. of Hawaii Manoa Campus, Honolulu, Hawaii.

Schizonts. Binucleated 4–6 μ in diameter; tetranucleated 6.6–11.0 μ in diameter, stained.

Sporonts. 5–7 μ in length, stained.

Spore. 5.0 × 2.8 μ, fresh state; 4.9 × 2.9 μ stained.

Polar filament 75–105 μ fresh state.

Holotype and 3 paratype slides deposited in the collection of the Center for Pathobiology, Univ. of California, Irvine, Calif. Additional paratype slides in author's collection.

Biology. The biology of *N. tephrititae* sp. n. was difficult to study since it is an intracellular parasite and most of its life cycle is completed within the cells of its host. Therefore, a logical sequence of the stages in the life cycle of *N. tephrititae* sp. n. was arranged by employing the present knowledge of microsporidian reproduction, physical alteration of the various stages and the chronological order in which infected larvae were sacrificed.

The emergence of the amoebula from the spore was not observed, but sections of diseased larvae indicated that the initial invasion and infection occurred in the midgut.

Within the host cells, the amoebula increased in size and became a spherical binucleated schizont approximately 4 μ in diameter (Fig. 1A). These Giemsa-stained schizonts had deep red nuclei and dense blue cytoplasm. The nuclei and the cytoplasm of these young schizonts appeared homogenous. Older binucleated schizonts were approximately 6 μ in diameter and the area immediately surrounding the nuclei was lightly stained (Fig. 1B, C). Schizonts nearing nuclear division had large nuclei (Fig. 1D) which, upon division, gave rise to tetranucleated schizonts which were approximately 6.6 μ in diameter (Fig. 1E). The cytoplasm stained densely around the periphery of the cell and lightly in the region of the dark staining nuclei. Some of the older tetranucleated schizonts measured up to 11 μ in diameter and were irregular in shape (Fig. 1F). The cytoplasm in these large schizonts stained light blue throughout and the large nuclei stained light red. After nuclear division, a cytoplasmic division occurred producing two binucleated daughter cells. Schizonts that were to become sporonts were approximately 6 to 7 μ in their widest dimensions, irregular in shape and had large hemispherical nuclei (Fig. 1G). Both cytoplasm and nuclei stained weakly. Early stage sporonts were definitely binucleated (Fig. 1H), but later, the nuclei lost their integrity and appeared as diffuse red staining streaks in the lightly stained, vacuolated cytoplasm (Fig. 1I). As the sporonts matured into spores, their nuclei seemed to dissipate and the cytoplasm began to condense.

TABLE 1. *Comparison of some of the characteristics of* Nosema *species recorded from Diptera with those of* Nosema tephrititae sp. n.

Pathogen	*Spore shape*	*Spore size* (μ) *Length*	*Width*	*Polar filament length* (μ)	*Sites of infection*	*Host*
Nosema aedis Kudo 1930	Pyriform	8.2 (7.5–9.0)	5.0 (4.0–6.0)	——	Fat body of larvae	*Aedes aegypti* (Culicidae)
Nosema bibionis Stammer 1956	Oval	4.2 (3.5–5.0)	2.8 (2.5–3.0)	——	Fat body	*Bibio varipes* (Bibionidae)
Nosema binucleatum Weissenberg 1926	Oval	5.5 (4.3–6.7)	2.8 (2.6–3.0)	60	Gut	*Tipula gigantea* (Tipulidae)
Nosema chapmani Kellen, Clark & Lindegren 1967	Elongate	5.5	1.7	60–70	Oenocytes	*Anopheles pseudopunctipennis franciscanus* (Culicidae)
Nosema kingi Kramer 1964	Oval	4.3	2.6	75–95	Fat body, gut, tracheae, muscle, malpighian tubules, reproductive tract	*Drosophila willistoni* (Drosophilidae)
Nosema lunatum Kellen, Clark & Lindegren 1967	Crecent	12.7	3.8	——	Oenocytes	*Culex tarsalis* (Culicidae)

TABLE 1. *Comparison of some of the characteristics of* Nosema *species recorded from Diptera with those of* Nosema tephrititae sp. n. (Cont.)

Pathogen	*Spore shape*	*Spore size* (μ) *Length*	*Width*	*Polar filament length* (μ)	*Sites of infection*	*Host*
Nosema micrococcus (Leger & Hesse 1921)	Spherical	——	1.9	——	Fat body of larvae	*Tanypus* (*Ablabechnia*) *setigera* (Chironomidae)
Nosema sphaeromiadis (Weiser 1957)	Oval	5.5	2.5	——	Fat body of larvae	*Sphaeromias* sp. (Ceratopogonidae)
Nosema stegomyiae Marchoux, Salimbeni, & Simond 1903	Reniform	5.5 (4.0–7.0)	2.5 (2.0–3.0)	50–60	Gut, muscle, air sac, fat body, malpighian tubules ovaries.	*Aedes aegypti* *Anophele gambiae* *A. melas* (Culicidae)
Nosema stricklandi Jirovec 1943	Pyriform	5.0	2.0	——	Fat body, giant cells	*Simulium* sp. (Simuliidae)
Nosema zavrreli Weiser 1946	Oval	3.5	2.0	——	Gut, epithelium	*Chironomus thumi* (Chironomidae)
Nosema tephrititae new species	Oval	5.0	2.8	75–105	Gut, tracheae, malpighian tubules, fat body, hemocyte, epidermis	*Dacus dorsalis* *D. cucurbitae* *Ceratitis capitata* (Tephritidae)

TABLE 2. *Size of spore of* Nosema tephrititae sp. n. *in fresh and stained preparations.*

Preparation	Number measured	Length (μ)			Width (μ)		
		Av. size	*St. dev.*	*Range*	*Av. size*	*St. dev.*	*Range*
Fresh[a]	100	5.0	0.2	4.2–6.0	2.8	0.2	2.0–3.0
Stained[b]	100	4.9	0.2	4.0–5.2	2.9	0.2	2.5–3.2

[a]Observations by phase-contrast microscope.
[b]Observations by ordinary light microscope.

As the spores matured, their spore walls became highly refractile. Although there were some variations, both live and stained spores appeared ovoid. In fresh preparations, spores averaged 5.0 μ in length and 2.8 μ in width (Table 2). Giemsa-stained spores (Fig. 1J) appeared about the same size as fresh spores averaging 4.9 μ in length and 2.9 μ in width. Macrospores present in some species of *Nosema* do not occur in *N. tephrititae* sp. n.

The polar filament (Fig. 1K) was easily forced out of the spore by applying pressure on the cover glass of a wet mount. Measurements were taken from 13 spores having their polar filament extruded in a relatively straight course. The lengths ranged from 75 to 105 μ with a mean of 88.5 μ and a standard deviation of 9.9 μ. Some of these polar filaments were tightly coiled when extruded from the spore indicating that the polar filament was tightly coiled within the spore as reported by Huger (1960).

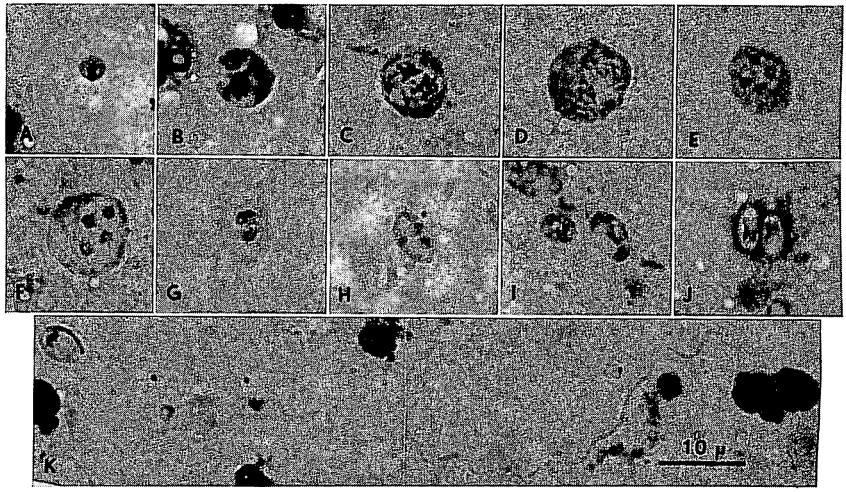

FIG. 1. Stages in the life cycle of *Nosema tephrititae* sp. n. A. Young binucleated schizont. B, C. Older binucleated schizonts. D. Binucleated schizont with nuclei dividing. E, F. Young and older tetranucleated schizonts. G. Binucleated schizont prior to sporont stage. H. Binucleated sporont. I. Sporont with diffused nuclei. J. Spores. K. Spore with extruded polar filament.

In addition, a few extruded polar filaments had a small body attached to the distal end which seems to represent the emerging amoebula (Kramer, 1960).

Symptomatology. The infection of the larvae of *D. dorsalis* by *N. tephtitae* sp. n. appeared to be asymptomatic. General external symptoms and signs, such as color change, dwarfness, distention, loss of appetite and sluggishness, characteristic of *Nosema* infections were not elicited by *N. tephritidae* sp. n. in *D. dorsalis* larvae. Externally there was no apparent way to distinguish diseased from healthy larvae. Moreover, no abnormalities of the internal organs were observed in diseased 3rd instar larvae. A 12- to 24-hour extension of the larval period appeared to be the only noticeable difference between normal and diseased larvae. Most of the larvae appeared to pupate normally when placed in the pupation medium.

The majority of the treated insects, however, died as pupae after the adult was formed in the puparium. The infection obviously fulminated after pupation since there was a tremendous increase both in the number of pathogens and in the number of cells attacked. *N. tephritidae* sp. n. apparently had its major effect after the host pupated, but even these pupae showed no obvious symptoms of nosemosis.

Infected adults, however, did show sufficient symptoms so that they could be distinguished in a cage full of healthy flies. Infected adults were sluggish and either could not fly or were able to fly only with diffi-

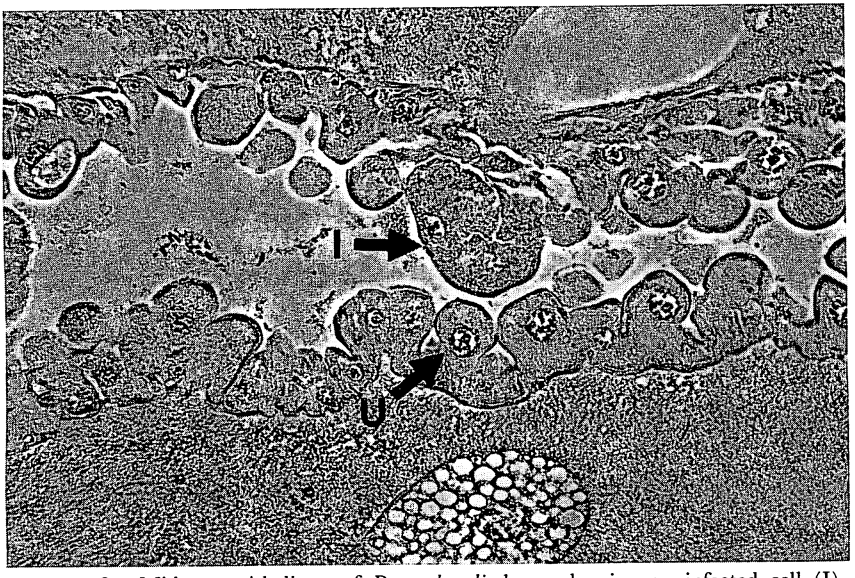

FIG. 2. Midgut epithelium of *Dacus dorsalis* larva showing an infected cell (I) hypertrophied due to excessive spore formation and an uninfected cell (U). ×150.

culty. Their abdomens were swollen, distended and appeared to be much whiter or paler than the abdomen of a normal adult. Their wings were held at a peculiar "droopy" angle.

The symptomatology of this *Nosema* infection, therefore, is the reverse of those usually reported for lepidopterans where the larvae show striking symptoms while the adults usually are symptomless (Lipa and Martignoni, 1960; Tanabe and Tamashiro, 1967).

Histopathology and Course of Infection. Table 3 summarizes the observations made to follow the course of infection in the larvae of *D. dorsalis*.

Since the oral route is the primary mode of entry for microsporidians, and since the midgut cells were susceptible to attack, it was not surprising to find the initial site of infection in the midgut (Fig. 2). The infection was first manifested by the presence of isolated cells in the midgut which were filled with *Nosema* spores. These infected cells were sparsely scattered throughout the midgut and were first detected 72 hours after the larvae were allowed to feed on the treated medium.

Although the infection was initially discovered in the 72-hour sections, it was apparent that the infection had to have occurred earlier since the

TABLE 3. *Observations of saggital sections and stained smears of* Dacus dorsalis *larvae following treatment with* Nosema tephrititae sp. n. *showing the time organs and tissues became infected.*

		Organs and tissues examined						
Time (hours)	*Stained smears*	*Mesenteron*	*Tracheal matrix*	*Fat body*	*Malpighian tubules*	*Muscle*	*Epidermis*	*Hemocytes*
72	a	+	−	−	−	−	−	−
78	a, b	+	−	+	+	+	−	−
84	a	+	−	−	−	−	−	−
90	a, b, c	+	−	−	−	−	−	−
96*	a, b, c							
102	a, b, c	+	−	−	+	+	−	−
108	a, b, c	+	+	−	−	+	+	+
114	a, b, c	+	+	+	−	+	−	−
120	a, b, c	+	−	−	+	+	−	−
128	a, b, c	+	−	+	+	+	−	−
134	a, b, c	+	+	+	−	+	−	+
140	a, b, c	+	+	+	+	−	−	+
146	a, b, c	+	+	+	−	+	−	+
152*	a, b, c							
158	a, b, c	+	+	−	−	+	+	+
164	a, b, c	+	+	+	−	+	+	+
170	a, b, c	+	+	+	−	+	−	+
176	a, b, c	+	+	+	+	+	−	+
182	a, b, c	+	+	+	+	+	+	+
188	a, b, c	+	+	+	+	+	+	+

a schizonts; b sporonts; c spores.
\+ infected; − uninfected.
* no sections made.

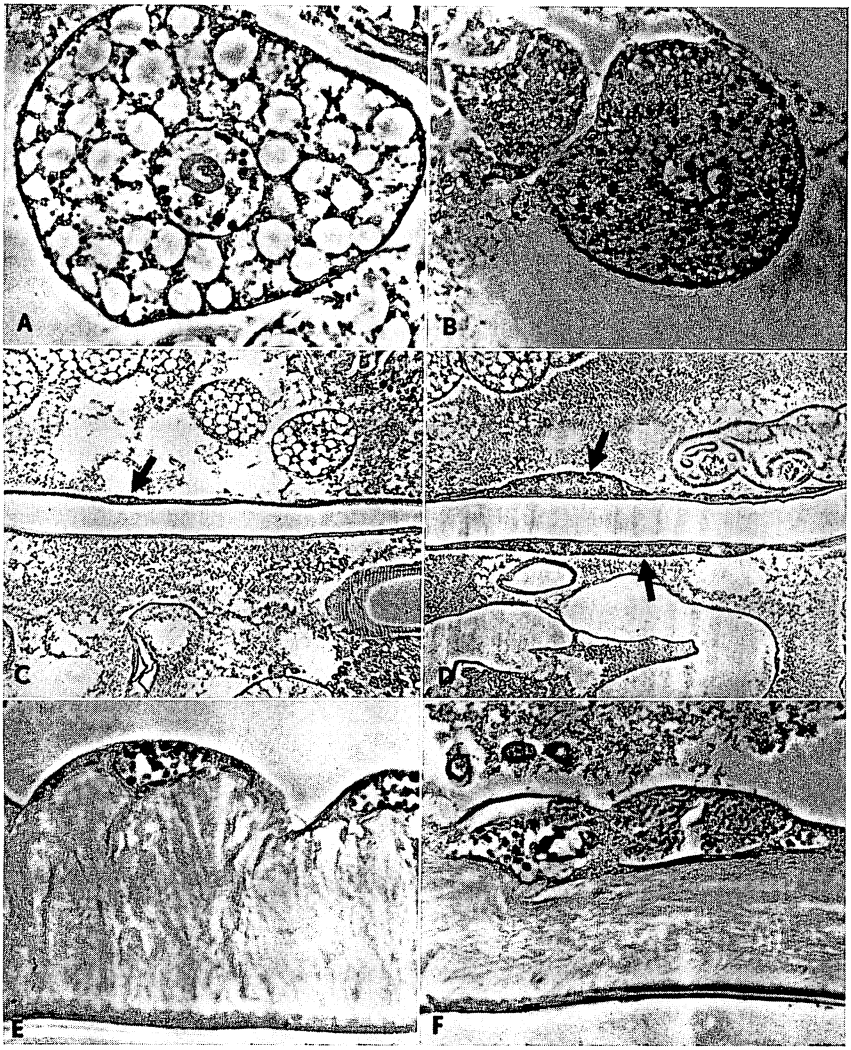

FIG. 3. Tissues of *Dacus dorsalis* larva showing both uninfected and infected with *Nosema tephritidae* sp. n. A. Uninfected fat cell. B. Infected fat cell filled with spores. C. Saggital section of trachea with uninfected tracheal cell (Arrow). D. Saggital section of infected trachea with hypertrophied tracheal cells (Arrows). E. Uninfected cell of integument. F. Infected cell of integument filled with spores. C, D, ×150 and all others, ×600.

protozoan had already sporulated in these cells. That the infection had started earlier was confirmed when schizonts were found in some 48-hour smears. The protozoan apparently completes its development in these initially invaded cells; *i.e.*, sporulates before breaking out of the cell to

spread the infection. This is surprising since one would normally not expect sporogony to start until the infection was well advanced with most of the susceptible tissues invaded. Therefore, because of the peculiarity in the multiplication of this pathogen, all of the developmental stages could be found early in the infection.

Apparently, those few cells that were initially attacked were invaded purely by chance since there were no apparent differences in those cells attacked and those that were not initially attacked. All of the cells of the midgut were susceptible to attack as shown later when the infection was well advanced. From these initial foci, the infection simultaneously spread throughout the midgut and into the hemocoel to the other susceptible tissues.

Except for 1 or 2 highly susceptible insects in which the infection progressed with abnormal speed, the tissue first attacked after the *Nosema* penetrated the hemocoel approximately 102 hours after treatment was the muscles. The *Nosema* only caused a slight enlargement of the muscles but apparently did initiate a breakdown in some of the individual fibers. This may in part explain the reasons for the droopy wing symptom in infected adults and the reason for the inability of the affected adults to fly.

Infections in the fat bodies (Fig. 3A, B), the hemocytes and the tracheal matrix (Fig. 3C, D) were generally found between 108 to 120 hours after treatment. The fat bodies, usually the first tissues attacked by many intracellular pathogens, surprisingly were not invaded until the infection

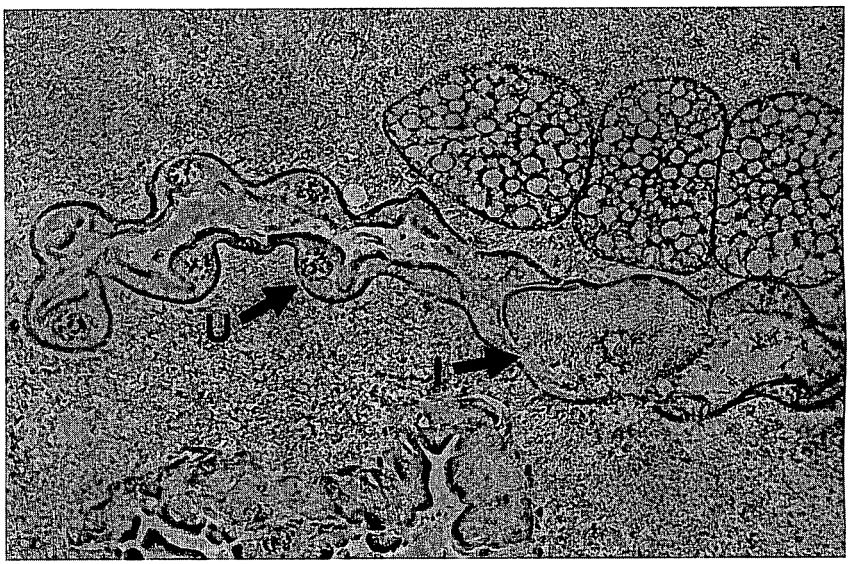

FIG. 4. Malpighian tubule of *Dacus dorsalis* larva showing an infected cell (I) hypertrophied due to excessive spore formation and an uninfected cell (U). ×150.

was well established in the mesenteron and muscles. The hemocytes which also are usually attacked early, were often found in large aggregations in the posterior parts of the larva. Other hemocytes were found intimately associated with diseased tissues. Spore-filled hemocytes were commonly found in almost all the larvae 134 hours after treatment. The tracheal matrix quickly became severely infected and the cells were greatly hypertrophied (Fig. 3C, D). In some larvae, the entire network of tracheal epithelia were infected.

The infections in the epidermis (Fig. 3E, F) and the Malpighian tubules (Fig. 4) did not occur with any consistency until 182 hours after treatment. Although the first infection in these tissues occurred as early as 102 hours, many of the larvae sectioned subsequent to this time did not show any signs of infections.

The epidermal infections were localized in relatively small areas in the caudal region of the larva. The infections became acute after 182 hours and most of the epidermal cells became infected causing a hypertrophy of the entire epidermis. In addition to the epidermal cells, portions of the endocuticula appeared to be disrupted by *N. tephrititae*. sp. n.

Although there were some unusual features in the course of attack by the *Nosema*, there was an even more unusual feature in the distribution of the infected tissues in the host. Surprisingly, only the susceptible tissues in the posterior two-thirds of the body were attacked. Although much of the susceptible tissues, such as the mesenteron and Malpighian tubules, are located in the posterior parts of the body, there were susceptible tissues, such as the epidermis, fat bodies, and muscles which were not attacked if they were located in the anterior parts of the host.

The reason for this regional selectivity is not completely understood, but it may be associated with the fact that there is a high concentration of blood cells in the posterior parts of many Diptera and almost none in the anterior region (Nappi and Stoffalano, 1972). If the blood cells play an important role in the spread of the *Nosema*, their absence in the anterior portion may account for the lack of infection. Or, if there is a mechanism that prevents the blood cells from being transported to the anterior region, it may also stop the schizonts from being carried forward since they are in the same size range as the blood cells. Alternatively, although it may appear unusual, the epidermis, muscles and fat bodies in the anterior regions may be less susceptible to attack than those in the rear.

Host Range. The host specificity of *N. tephrititae* sp. n. tested using several dipterous and one lepidopterous species. Dipterans closely related to *D. dorsalis*, namely, *D. cucurbitae* Coquillet and *Ceratitis capitata* Weidemann were susceptible to *N. tephrititae* sp. n. The symptoms elicited by the protozoan in these hosts were similar to those in *Dacus dorsalis*. Unrelated species, such as *Musca domestica* L. and *Drosophila immigrans* Sturtevant,

were also susceptible to the *Nosema*. The majority of the treated larvae pupated and died as pupae although a few did emerge as adults. These adults appeared normal but were sluggish when compared with the untreated controls. Wet mounts made from these treated adults showed that they were filled with the spores and vegetative stages of the pathogen.

Culex pipiens quiquefasciatus Say the only nematocerous fly tested was not susceptible to *N. tephrititae* sp. n. The 1st instar larvae consumed many spores, but stained smears of these individuals and of others after they had molted in later instars did not contain any vegetative stages of the pathogen. A few spores were also found in smears of adults and pupae, but again there were no vegetative stages of the pathogen. Adults of both test and control groups emerged at approximately the same time.

The lepidopteran tested was the lawn armyworm, *Spodoptera mauritia acronytoides* (Gueneé). First instar larvae, the stage most susceptible to *Nosema*, were allowed to feed on treated napier grass (*Pennisetum purpureum* Schumach). After allowing these caterpillars to feed for 72 hours, a few were sacrificed and examined for the presence of the pathogen. Although a few spores were present in stained preparations, infection was not apparent since the vegetative stages of the pathogen were not present. The larvae appeared normal and showed no sign of infection.

Summary

The biology and morphology of a previously undescribed species of *Nosema* pathogenic to the Oriental fruit fly, *Dacus dorsalis* Hendel, are described. *Nosema tephrititae* sp. n. is proposed for this new Microsporidian. Pathogenic effects and the course of infection in the host were determined. Initial invasion occurred in the cells of the midgut; the second tissue attacked was the skeletal muscles. Other tissues infected early include the fat bodies, tracheal matrix and the hemocytes. After hemocyte infection, the tracheal matrix and epidermis became infected. Diseased larvae and pupae appeared normal externally when compared to healthy individuals. Internally, hypertrophy of severely infected host cells was the most striking characteristic of the disease. The pathogen had its major effects on the pupae since treated larvae pupated but failed to emerge as adults. Sublethal doses of *Nosema* spores resulted in diseased adults which appeared sluggish with distended abdomen appearing paler than normal individuals. Larvae of the mosquito, *Culex quinquefasciatus* Say and the lawn armyworm, *Spodoptera mauritia acronytoides* (Gueneé), did not become infected when fed a massive number of spores; however, the larvae of the melon fly, *Dacus cucurbitae* Coquillett, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), the house fly, *Musca domestica* L., and *Drosophila immigrans* Sturtevant acquired subacute infection.

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